Carpenter, J. M. and Pierpoint, W. S. 1981. Cross-linking of the Subunits of Potato Virus X with Di-imidates. *Journal of General Virology.* 54 (2), pp. 391-395.

J. gen. Virol. (1981), **54**, 391–395
Printed in Great Britain

Cross-linking of the Subunits of Potato Virus X with Di-imidates

(*Accepted 23 February 1981*)

SUMMARY

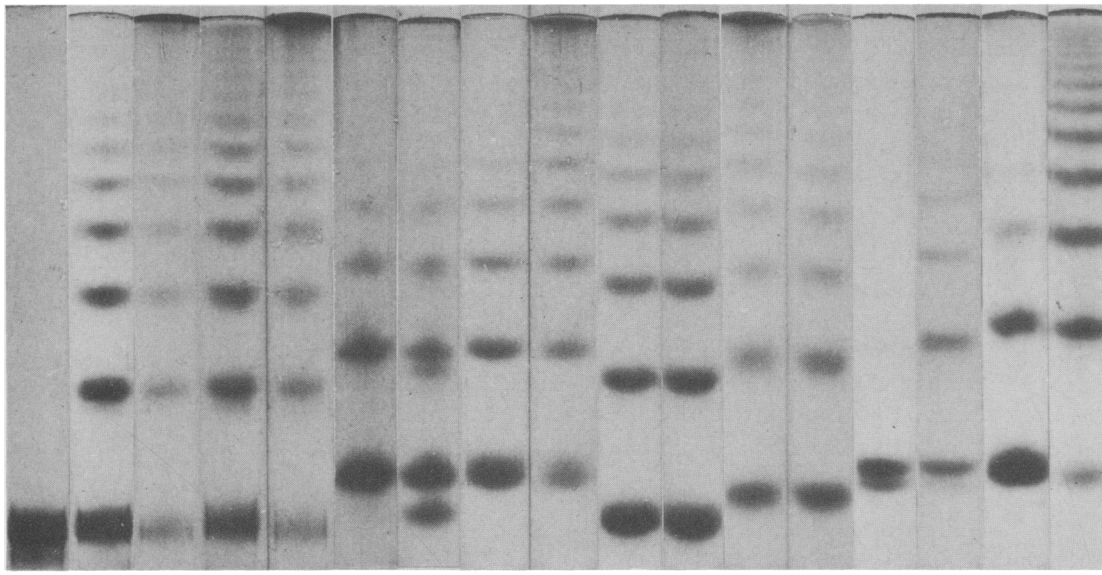
The protein subunits of the X^N strain of potato virus X (PVX) were cross-linked by di-imidates to produce a series of polymers in the proportion expected from the random cross-linking of subunits in an extensive heterologous array. Polymerization of the subunits of chemically modified forms of the virus, as well as of other strains, suggests that the cross-links are heterogeneous, and may, but need not, involve the two amino groups that have been previously characterized as either reacting with chlorogenoquinone or being sensitive to trypsin.

There are two regions associated with lysine residues in the protein subunits of intact potato virus X (PVX) particles that can be modified in extracts of leaves without necessarily destroying infectivity. One is a peptide link adjacent to a lysine residue which can be split by leaf proteases (Pierpoint *et al.*, 1977; Koenig *et al.*, 1978). The other is the E-amino group of a different lysine residue that reacts with *o*-quinones generated during the enzymic oxidation of leaf phenols (Pierpoint *et al.*, 1977). Both these reactions can occur and modify PVX during its extraction from infected leaves, but it is not clear whether they occur naturally in growing or senescing leaves (e.g. Ireland & Pierpoint, 1980). The two exposed lysine residues also react *in vitro* with synthetic reagents which modify amino groups; for example, both react partially with pyridoxal-5′-phosphate (Pierpoint & Carpenter, 1978). The remaining 9 to 10 lysine amino groups in the protein subunits, however, either do not appreciably react with these reagents (Pierpoint & Carpenter, 1978), or if they do, the reaction leads to virion disintegration.

This paper describes the reaction of PVX with di-imidates, bifunctional reagents which can cross-link suitably placed amino groups (Davies & Stark, 1970). Such reagents cross-link the protein subunits of cowpea chlorotic mottle and brome mosaic viruses (Bancroft & Smith, 1975) and also those of southern bean mosaic virus (Sehgal & Hsu, 1977), but there are no published studies of their effect on helical viruses. It was thought that the reagents might react preferentially with the two reactive amino groups of PVX and that they might detect some pattern in the arrangement of amino groups or subunits in PVX as they reveal patterns in the arrangement of subunits in polymeric enzymes (Hajdu *et al.*, 1976).

Three strains of PVX, X^N and X^4 (Ireland & Pierpoint, 1980) and X_{HB} (Moreira *et al.*, 1980) were cultured in *Nicotiana tabacum* var. Xanthi nc, and were isolated, their concentration estimated, and their infectivity measured as described previously (Pierpoint *et. al.*, 1977). Virus was diluted to contain approx. 1 mg (0·037 μmol protein subunits) in 1 ml disodium borate (0·15 M, pH 7·5 to 9·5) containing 11 μmol of either dimethyl suberimidate (DMS), dimethyl adipimidate (DMA; both from Pierce & Warringer Ltd., Chester, Cheshire, U.K. or diethyl malonimidate (DEM; synthesized as described by McElvain & Schroder, 1949). After incubation at 25 °C, remaining di-imidate was destroyed with hydroxylamine–HCl (22 μmol in 0·1 ml phosphate buffer pH 7) and samples (0·1 ml) were taken for analysis. These were treated with urea, 2-mercaptoethanol and SDS and subjected to polyacrylamide gel electrophoresis (PAGE) in SDS-containing 5% polyacrylamide gels (0·13% bis-acrylamide, 0·05 M-phosphate buffer pH 7·1; Carpenter *et al.*, 1977). Gels were stained with Coomassie Brilliant Blue and quantified by scanning with a Joyce-Loebl microdensitometer

0022-1317/81/0000-4540 $02.00



(*a*) (*b*) (*c*) (*d*) (*e*) (*f*) (*g*) (*h*) (*i*) (*j*) (*k*) (*l*) (*m*) (*n*) (*o*) (*p*) (*q*)

Fig. 1. Reaction of PVX with di-imidates. The X^N strain of PVX was examined by PAGE before (*a*) and after reaction with DMS at (*b*) pH 8·5 and (*c*) pH 9·5. The product at pH 8·5 was sedimented, resuspended and reacted a second time at (*d*) pH 8·5 and (*e*) pH 9·5. Another preparation at pH 8·5 (*f*) was treated briefly with trypsin (*g*). X^N was also reacted with DMA at (*h*) pH 8·5 and (*i*) pH 9·5, and with DEM at (*j*) pH 8·5 and (*k*) pH 9·5. The protein from X^N was examined after reaction (*l*) with DMS and (*m*) with DEM at pH 8·5. Pyridoxylated PVX was similarly examined before (*n*) and after (*o*) reaction with DMS at pH 8·5. The X^4 strain of PVX was examined after reaction with DMS at (*p*) pH 8·5 and (*q*) pH 9·5. Before reaction it gave a single band on PAGE similar to (*a*).

(Pierpoint *et al.*, 1977). Virus was briefly exposed to trypsin as described previously (Pierpoint *et al.*, 1977).

Incubating PVX-X^N with DMS at pH 8·5 for 1 to 2 h, decreased its infectivity to about half, and cross-linked 40 to 60% of its subunits into polymers the largest of which varied with experiment from hexamers up to nonomers (Fig. 1*b*). Cross-linked subunits were present in treated virus after sedimenting and are therefore unlikely to have been produced from disaggregated protein. The extent of cross-linking was increased only slightly when treated virus was sedimented, resuspended and retreated (Fig. 1*d*), or when it was subjected to three additions of DMS without being sedimented between treatments. The proportion of subunits that were polymerized also increased only slightly at a fourfold higher concentration of DMS, but decreased to 20% and 10% when the DMS concentration was lowered to 5·5 and 2·2 μmol/ml respectively; it was not appreciably affected by a 100-fold change (7 to 0·07 mg/ml) in the concentration of virus. Cross-linking was complete after 30 min of reaction (Fig. 2) and the proportion of each polymer was, with the possible exception of the dimers, constant for the next 3 h. The inset to Fig. 2 shows that the amount of each polymer formed after 3 h bears a logarithmic relationship to the composition of the polymer. This is characteristically the result of cross-links formed between non-identical groups on the subunits of large heterologous (Monod *et al.*, 1965) polymers in conditions where each additional cross-link only adds single subunits to cross-linked segments (Hajdu *et al.*, 1976; F. Bartha, personal communication). There was no indication of groups of subunits which were preferentially linked.

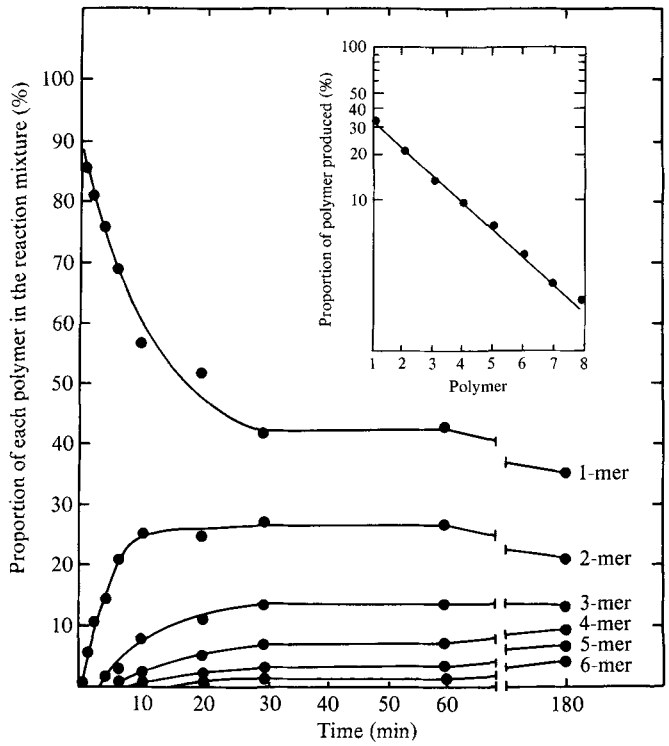


Fig. 2. Time course of the formation of cross-linked polymers of PVX subunits. Cross-linking was performed with DMS at pH 8·5 and samples analysed by PAGE. Inset shows the semi-logarithmic relationship between the composition of a polymer and the proportion of the final reaction mixture (180 min) that it comprises.

The degree of cross-linking depends markedly on pH; little or no cross-linking occurred at pH 7 to 7·5, while the reaction at pH 9·5 was so extensive that most (87%) monomer disappeared and 40 to 50% of it was converted to polymers which were too large to enter the gels (Fig. 1*c*). A time course study showed that polymerization at pH 9·5 was complete after 30 min; after 8 min the semi-logarithmic relation between polymer composition and amount formed was no longer linear, probably because in these conditions, new cross-links can join extensively polymerized segments (Hajdu *et al.*, 1976). The more extensive reaction at pH 9·5 was not a consequence of the reaction of disaggregated protein, for PVX is, in the ultracentrifuge, as stable in borate buffer at pH 9·5 as at 8·5 and 7·5, and its stability is not affected by DMS. Virus which was cross-linked at pH 8·5 was more extensively cross-linked when it was sedimented, resuspended and re-treated at pH 9·5 (Fig. 1*e*). Cross-linking at pH 9·5 decreased the infectivity of virus to 10% of that of control PVX kept at the same pH without added DMS: this decrease may well be due to the difficulty of uncoating the modified virus *in vivo* (Bancroft & Smith, 1975).

That more than one type of cross-link exists in the polymers is indicated by the effect on them of brief exposure to trypsin. This treatment completely converts native PVX protein to a form with a mol. wt. of about 25000 (Pierpoint *et al.*, 1977; Koenig *et al.*, 1978) but degrades about half of each PVX polymer so that it appeared as a duplex (Fig. 1*f*, *g*). Each polymer is thus a mixture of at least two isomers in which the components are linked in different ways: in one, the trypsin-sensitive site is unmodified, whereas in another it is either modified or sterically protected from trypsin attack. That the cross-links are also heterogeneous in size is

suggested by the different degrees of polymerization produced by other di-imidates. DMA whose maximum length is 1 nm (de Abreu *et al.*, 1979) polymerized protein subunits in a similar way to the slightly longer (1·1 nm) DMS (Fig. 1 *h, i*). DEM, only 0·5 nm long, links less effectively, and even at high concentrations and pH 9·5, produced only small amounts of polymer too large to enter the gels (Fig. 1 *j, k*).

Dissociated PVX protein has more reactive amino groups than does the protein of the virion (Pierpoint & Carpenter, 1978), and cross-links readily. Although the reaction with both DMS and DEM was negligible at pH 7·5, with DMS at pH 8·5 20% of polymers formed were too large to enter the gels (Fig. 1 *l, m*). Lowering the concentration of PVX protein from 2·9 to 0·06 mg/ml did not affect the extent of cross-linking nor the pattern of polymers appreciably more than a similar dilution affected the cross-linking of intact PVX. As dilution is expected to affect cross-linking between unassociated proteins more than between associated proteins (Davies & Stark, 1970) this may indicate a loose association between molecules of the isolated PVX protein. Other evidence (Goodman, 1975) suggests a limited, reversible association between subunits, although association appears to depend on the method of preparation and the evidence relates to protein isolated by methods other than that used here.

That the polymers derived from the virion contain more than one type of cross-link and that the cross-links need not involve the amino group of the trypsin-sensitive site, was confirmed by the reaction of di-imidates with forms or strains of PVX in which some amino groups were modified.

(i) A form of PVX which had been modified by plant proteases and which probably corresponds to trypsin-modified PVX (e.g. Koenig *et al.*, 1978) occurs in small amounts in some preparations of PVX. Although it has probably lost the trypsin-sensitive lysine, it is cross-linked by DMS to give dimers and trimers which migrate slightly faster than the corresponding polymers formed from unmodified virus (not illustrated).

(ii) Pyridoxylated PVX (Pierpoint & Carpenter, 1978) contains two types of protein which are pyridoxylated either on the trypsin-sensitive site or the quinone-reactive site. It can be cross-linked by di-imides much as is PVX, although generally less extensively. Although the two forms of monomeric protein are poorly resolved in SDS–PAGE it is clear that the faster-moving, trypsin-sensitive form (Pierpoint & Carpenter, 1978) is preferentially polymerized (Fig. 1 *n, o*).

(iii) PVX strains X^4 and X_{HB} (Moreira *et al.*, 1980) are insensitive to brief exposure to trypsin (J. M. Carpenter & W. S. Pierpoint, unpublished results) and presumably do not contain the trypsin-sensitive site. They are, nevertheless, cross-linked by DMS and DMA although not so extensively as the X^N strain (Fig. 1 *p, q*). Cross-linking was diminished but by no means abolished after pyridoxylation of these strains so as to block the quinone-reactive site.

With X^4, as with the X^N strain, DEM produced relatively little cross-linking. With X_{HB}, however, especially at pH 9·5 it was as effective as DMS, suggesting that this strain has more amino groups in adjacent subunits that are closer than 0·5 nm.

Thus, the protein subunits of PVX are cross-linked by di-imidates as if they were part of a heterologous array and the cross-links may, but need not, involve the amino groups at the trypsin-sensitive and the quinone-reactive sites. The subunits contain too many amino groups which react extensively with di-imidates to reveal any pattern in their arrangement in the virus, and this indicates a limitation to the use of di-imidates in analysing virus structure at this level. Cross-linking occurs more extensively at pH 9·5 than at pH 8·5, as it does with southern bean mosaic virus (Sehgal & Hsu, 1977) and is not a result of virion dissociation at higher pH values nor the instability of di-imidates at lower pH values (Browne & Kent, 1975). It may partly depend on the two different mechanisms by which di-imidates react with amino groups (Browne & Kent, 1975; Peters & Richards, 1977) and which are more likely to result

in cross-links at higher pH values, although if this is so it is surprising that more cross-links can be introduced into virus treated at pH 8·5 by re-treating it at pH 9·5. Alternatively, the difference between cross-linking at the two pH values may depend on more unprotonated amino groups being reactive to di-imidates at pH 9·5; but although PVX contains some protons which dissociate between pH 8·5 and 9·5, they are less than 1 per subunit (Shaw, 1977).

Rothamsted Experimental Station J. M. CARPENTER
Harpenden, Herts., U.K. W. S. PIERPOINT*

REFERENCES

BANCROFT, J. B. & SMITH, D. B. (1975). The effect of dimethyl-adipimidate on the stability of cowpea chlorotic mottle and brome mosaic virus. *Journal of General Virology* **27**, 257–261.

BROWNE, D. T. & KENT, S. B. H. (1975). Formation of non-amidine products in the chemical modification of horse liver alcohol dehydrogenase with imido esters. *Biochemical and Biophysical Research Communications* **67**, 133–138.

CARPENTER, J. M., KASSANIS, B. & WHITE, R. F. (1977). The protein and nucleic acid of beet yellows virus. *Virology* **77**, 101–109.

DAVIES, G. E. & STARK, G. R. (1970). Use of dimethyl suberimidate, a cross-linking reagent, in studying the subunit structure of oligomeric proteins. *Proceedings of the National Academy of Sciences of the United States of America* **66**, 651–656.

DE ABREU, R. A., DE VRIES, J., DE KOK, A. & VEEGER, C. (1979). Cross-linking studies with the pyruvate dehydrogenase complexes from *Azotobacter vinelandii* and *Escherichia coli*. *European Journal of Biochemistry* **97**, 379–387.

GOODMAN, R. M. (1975). Reconstruction of potato virus X *in vitro*. I. Properties of dissociated protein structural subunits. *Virology* **68**, 287–298.

HAJDU, J., BARTHA, F. & FRIEDRICH, P. (1976). Cross-linking with bifunctional reagents are a means for studying the symmetry of oligomeric proteins. *European Journal of Biochemistry* **68**, 373–383.

IRELAND, R. J. & PIERPOINT, W. S. (1980). Reaction of strains of potato virus X with chlorogenoquinone *in vitro*, and the detection of a naturally modified form of the virus. *Physiological Plant Pathology* **16**, 81–92.

KOENIG, R., TREMAINE, J. H. & SHEPARD, J. F. (1978). In situ degradation of the protein chain of potato virus X at the N- and C-termini. *Journal of General Virology* **38**, 329–337.

McELVAIN, S. M. & SCHROEDER, J. P. (1949). Orthoesters and related compounds from malono-succinonitriles. *Journal of the American Chemical Society* **71**, 40–46.

MONOD, J., WYMAN, J. & CHANGEUX, J-P. (1965). On the nature of allosteric transitions: a plausible model. *Journal of Molecular Biology* **12**, 88–118.

MOREIRA, A., JONES, R. A. C. & FRIBOURG, C. E. (1980). Properties of a resistance breaking strain of potato virus X. *Annals of Applied Biology* **95**, 93–103.

PETERS, K. & RICHARDS, F. M. (1977). Chemical cross-linking: reagents and problems in studies of membrane structure. *Annual Review of Biochemistry* **46**, 523–551.

PIERPOINT, W. S. & CARPENTER, J. M. (1978). An infective pyridoxal-derivative of potato virus X: PVX-PLP. *Journal of General Virology* **38**, 505–518.

PIERPOINT, W. S., IRELAND, R. J. & CARPENTER, J. M. (1977). Modification of proteins during the oxidation of leaf phenols: reaction of potato virus X with chlorogenoquinone. *Phytochemistry* **16**, 29–34.

SEHGAL, O. P. & HSU, C. H. (1977). Identity and location of a minor protein component in virions of southern bean mosaic virus. *Virology* **77**, 1–11.

SHAW, J. (1977). Hydrogen-ion titration of potato virus X protein. *Journal of General Virology* **36**, 357–360.

(Received 12 December 1980)